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## **The ammonia transporter RhCG modulates urinary acidification by interacting with the vacuolar proton-ATPases in renal intercalated cells**

Bourgeois, Soline ; Bounoure, Lisa ; Mouro-Chanteloup, Isabelle ; Colin, Yves ; Brown, Dennis ; Wagner, Carsten A

**Abstract:** Ammonium, stemming from renal ammoniagenesis, is a major urinary proton buffer and is excreted along the collecting duct. This process depends on the concomitant secretion of ammonia by the ammonia channel RhCG and of protons by the vacuolar-type proton-ATPase pump. Thus, urinary ammonium content and urinary acidification are tightly linked. However, mice lacking Rhcg excrete more alkaline urine despite lower urinary ammonium, suggesting an unexpected role of Rhcg in urinary acidification. RhCG and the B1 and B2 proton-ATPase subunits could be co-immunoprecipitated from kidney. In ex vivo microperfused cortical collecting ducts (CCD) proton-ATPase activity was drastically reduced in the absence of Rhcg. Conversely, overexpression of RhCG in HEK293 cells resulted in higher proton secretion rates and increased B1 proton-ATPase mRNA expression. However, in kidneys from Rhcg<sup>-/-</sup> mice the expression of only B1 and B2 subunits was altered. Immunolocalization of proton-ATPase subunits together with immuno-gold detection of the A proton-ATPase subunit showed similar localization and density of staining in kidneys from Rhcg<sup>+/+</sup> and Rhcg<sup>-/-</sup> mice. In order to test for a reciprocal effect of intercalated cell proton-ATPases on Rhcg activity, we assessed Rhcg and proton-ATPase activities in microperfused CCD from Atp6v1b1<sup>-/-</sup> mice and showed reduced proton-ATPase activity without altering Rhcg activity. Thus, RhCG and proton-ATPase are located within the same cellular protein complex. RhCG may modulate proton-ATPase function and urinary acidification, whereas proton-ATPase activity does not affect RhCG function. This mechanism may help to coordinate ammonia and proton secretion beyond physicochemical driving forces.

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**The ammonia transporter RhCG regulates urinary acidification by  
interacting with vacuolar H<sup>+</sup>-ATPases  
in renal intercalated cells**

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Running title: *Rhcg regulates H<sup>+</sup>-ATPases in intercalated cells*

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## ABSTRACT

Ammonium, stemming from renal ammoniogenesis, is a major urinary proton buffer and is excreted along the collecting duct. This process depends on the concomitant secretion of ammonia (NH<sub>3</sub>) by the NH<sub>3</sub> channel RhCG and of protons (H<sup>+</sup>) by the vacuolar-type H<sup>+</sup>-ATPase pump. Thus, urinary ammonium content and urinary acidification are tightly linked. However, mice lacking Rhcg excrete more alkaline urine despite lower urinary ammonium, suggesting an unexpected role of Rhcg in urinary acidification.

RhCG and the B1 and B2 H<sup>+</sup>-ATPase subunits could be coimmunoprecipitated from kidney. In *ex vivo* microperfused cortical collecting ducts (CCD) H<sup>+</sup>-ATPase activity was drastically reduced in the absence of Rhcg. Conversely, overexpression of RhCG in HEK293 cells resulted in higher H<sup>+</sup> secretion rates and increased B1 H<sup>+</sup>-ATPase mRNA expression. However, in kidneys from *Rhcg*<sup>-/-</sup> mice the expression of only B1 and B2 subunits was altered. Immunolocalization of H<sup>+</sup>-ATPase subunits together with immuno-gold detection of the A H<sup>+</sup>-ATPase subunit showed similar localization and density of staining in kidneys from *Rhcg*<sup>+/+</sup> and *Rhcg*<sup>-/-</sup> mice. In order to test also for a reciprocal effect of intercalated cell H<sup>+</sup>-ATPases on Rhcg activity, we assessed Rhcg and H<sup>+</sup>-ATPase activities in mCCD from *ATP6v1B1*<sup>-/-</sup> mice and showed reduced H<sup>+</sup>-ATPase activity without altering Rhcg activity.

Thus, RhCG and H<sup>+</sup>-ATPase are located within the same cellular protein complex. RhCG may modulate H<sup>+</sup>-ATPase function and urinary acidification, whereas H<sup>+</sup>-ATPase activity does not affect RhCG function. This mechanism may help to coordinate NH<sub>3</sub> and H<sup>+</sup> secretion beyond the physico-chemical driving forces.

## INTRODUCTION

The renal collecting duct excretes acid into urine by parallel secretion of protons ( $H^+$ ) and ammonia ( $NH_3$ ) by vacuolar  $H^+$ -ATPases ( $H^+$ -ATPase) and the ammonia transporter RhCG<sup>1-3</sup>. Renal acid excretion is critical for maintaining normal acid-base homeostasis and is increased during acidosis or following a systemic acid-load<sup>2</sup>.

Highly conserved through evolution from bacteria to humans, RhCG belongs to the Rh (Rhesus) glycoprotein family and has been characterized by *in vitro* and *ex vivo* studies as a channel selectively permeable to  $NH_3$  but not to ammonium ( $NH_4^+$ )<sup>4-6</sup>. In the kidney, RhCG expression specifically spreads from the late distal convoluted tubule (DCT) to the inner medullary collecting duct (IMCD), on both basolateral and apical poles of most epithelial cells (with the exception of selective apical expression in non-type A intercalated cells)<sup>7-11</sup>. We recently showed that RhCG is responsible for a major part of  $NH_3$  transported through apical and basolateral membranes of the collecting duct resulting in a drastic reduction of transepithelial  $NH_3$  transport in collecting ducts from *Rhcg*<sup>-/-</sup> mice<sup>11-12</sup>. As a consequence of this defect, chronically acid-loaded *Rhcg*<sup>-/-</sup> mice cannot eliminate  $NH_4^+$  into urine and develop a severe incomplete distal renal tubular acidosis (dRTA) characterized by low blood pH and  $HCO_3^-$  concentration<sup>11-12</sup>. Similarly, mice with selective deletion of *Rhcg* from only the collecting duct or intercalated cells show reduced urinary ammonium excretion<sup>13-14</sup>.

While RhCG is responsible for luminal  $NH_3$  secretion, the renal  $H^+$ -ATPase is critical to actively secrete  $H^+$  into urine<sup>15-16</sup>.  $H^+$ -ATPases are ubiquitous multi-subunits proteins composed of a cytosolic  $V_1$  catalytic domain, responsible for ATP hydrolysis and a membrane-associated  $V_0$  domain mediating  $H^+$  translocation<sup>16-17</sup>. In the collecting duct,  $H^+$ -ATPases are localized at the luminal side of type-A intercalated cells and basolateral side of non-type A intercalated cells. The  $V_1$  domain contains 8 subunits A-H including the tissue-specific subunit isoforms B1 and B2. The B1 isoform is highly enriched in intercalated cells of kidney and its genetic mutation or deletion cause a form of distal renal tubular acidosis<sup>18-20</sup>. The  $V_0$  domain contains 6 different subunits, a, d, c, c', c'', and e including a1, a2, a3 and a4. Also, the a4 isoform is highly expressed in kidney<sup>21-22</sup> and mutations in human and its deletion in mice are associated with renal tubular acidosis<sup>23-25</sup>.

Urinary acidification and ammonium content are closely linked<sup>26</sup>. Secretion of  $\text{NH}_3$  and  $\text{H}^+$  by RhCG and  $\text{H}^+$ -ATPases may be coordinated since protonation of  $\text{NH}_3$  and subsequent trapping of  $\text{NH}_4^+$  are critical for the efficient excretion of  $\text{NH}_4^+$ <sup>26-29</sup>. The partial overlap of both proteins in type-A intercalated cells may allow even for a direct functional coupling or interaction<sup>30</sup>. We have recently shown in two differently constructed mouse models lacking Rhcg that urinary pH is more alkaline during stimulation of urinary acidification and  $\text{NH}_4^+$  excretion<sup>11-12</sup>. Since *Rhcg*<sup>-/-</sup> mice have highly reduced  $\text{NH}_3$  secretion, the lack of  $\text{NH}_3$  buffering capacity would be expected to result in a rather more acidic urinary pH, as shown in another mouse model with intact Rhcg-mediated  $\text{NH}_3$  secretion but reduced medullary  $\text{NH}_4^+$  accumulation<sup>31</sup>. Thus, we hypothesized that the absence of Rhcg may reduce proton secretion by  $\text{H}^+$ -ATPases.

## RESULTS

### *Rhcg and B1 and B2 subunits of H<sup>+</sup>-ATPase are part of one protein complex in native kidney*

To assess whether RhCG and H<sup>+</sup>-ATPase were located within the same cellular protein complex in intercalated cells we performed coimmunoprecipitation of RhCG and B1 and B2 H<sup>+</sup>-ATPase subunits from native rat kidney medulla (Figure 1). Immunoprecipitation with antibodies against RhCG or B1 (panel A and B) or RhCG or B2 (panel C and D) after chemical crosslinking yielded protein complexes that contained both proteins whereas immunoprecipitation with control IgG did not detect RhCG or the B subunits. Thus, RhCG, B1 and B2 containing H<sup>+</sup>-ATPases are part of the same protein complex in kidney.

We further confirmed partial colocalization of Rhcg and B1 subunits at the apical pole of intercalated cells in mouse kidney by immunohistochemistry (Figure 1, panel E).

### *Rhcg<sup>-/-</sup> mice show a reduced capacity to excrete H<sup>+</sup> from kidney CCD cells*

We recently showed in *ex vivo* microperfused collecting ducts from *Rhcg<sup>-/-</sup>* mice a reduced NH<sub>3</sub> transepithelial flux due to decrease in apical and basolateral NH<sub>3</sub> permeabilities<sup>11-12</sup>. Here, using the same approach, we assessed H<sup>+</sup> transport activity in microperfused CCDs from *Rhcg<sup>+/+</sup>* and *Rhcg<sup>-/-</sup>* mice challenged for 2 days with an HCl load in food to maximize transport activities. Luminal NH<sub>4</sub>Cl prepulses were performed and H<sup>+</sup> transport activity was assessed from the steep pH<sub>i</sub> recovery rates after maximal intracellular acidification when NH<sub>4</sub>Cl was removed from the lumen<sup>32-33</sup>. The initial steep rate of alkalinisation reflects H<sup>+</sup> extrusion, mostly by H<sup>+</sup>-ATPases<sup>33</sup> (Figures 2A and 2B). We also observed in WT mice an early, almost immediate but slow pH<sub>i</sub> recovery which was not observed in KO mice suggesting that it is related to the function of Rhcg. As the initial pH<sub>i</sub> values were slightly different in CCDs from *Rhcg<sup>-/-</sup>* and *Rhcg<sup>+/+</sup>* mice, we also measured intracellular buffering power and calculated H<sup>+</sup> fluxes (JH<sup>+</sup>) across the membrane to directly compare transport rates. Similar to pH<sub>i</sub> recovery rates, JH<sup>+</sup> was greatly reduced in CCDs from *Rhcg<sup>-/-</sup>* by 89% (*Rhcg<sup>+/+</sup>*: 32.6 ± 12.8 pmol/mm/min vs. *Rhcg<sup>-/-</sup>*: 3.6 ± 0.63 pmol/mm/min, p ≤ 0.0001) (Figure 2C). Thus, the more alkaline urine observed in *Rhcg<sup>-/-</sup>* during acid-loading results from impaired proton secretion along the collecting duct.

### *The absence of Rhcg alters B1 and B2-H<sup>+</sup>-ATPase subunits expression*

Next, we investigated whether the reduced H<sup>+</sup> flux observed in microperfused CCDs from *Rhcg*<sup>-/-</sup> mice could be linked to altered expression of H<sup>+</sup>-ATPase subunits. At the mRNA level, we could not detect any variation of the intercalated cell-enriched B1 H<sup>+</sup>-ATPase isoform (suppl. figure 1A), and the more ubiquitous B2 (suppl. Figure 1B) and a4 (suppl. Figure 1C) H<sup>+</sup>-ATPase isoforms between *Rhcg*<sup>+/+</sup> and <sup>-/-</sup> mice. In membrane fractions from medullary kidney tissue to enrich the proportion of H<sup>+</sup>-ATPases originating from intercalated cells, the amount of the intercalated cell specific B1 isoform (Figure 3A) was increased whereas the B2 isoform (Figure 3B) was decreased in kidney tissue from *Rhcg*<sup>-/-</sup> mice (p ≤ 0.01). However, the protein abundance of the ubiquitous E2 (Figure 3C) and a1 isoforms (Figure 3D) as well as the kidney-enriched a4 (Figure 3E) subunits of the H<sup>+</sup>-ATPase was not different between the 2 genotypes.

### *Rhcg does not regulate H<sup>+</sup>-ATPase localization*

Reduced H<sup>+</sup>-ATPase activity may result from altered subcellular localization of H<sup>+</sup>-ATPases. Immunostaining for the B1, B2, E and a4 H<sup>+</sup>-ATPase subunits showed no apparent difference in subcellular localization of the 3 proteins in type-A intercalated cells in *Rhcg*<sup>+/+</sup> and *Rhcg*<sup>-/-</sup> kidneys (Figure 4 and suppl. Figure 2). Electron microscopy with immunogold staining for the ubiquitous A subunit similarly detected no difference in the subcellular localization of this H<sup>+</sup>-ATPase subunit (Figure 5). The absence of Rhcg did not affect the immunoreactivity for the A subunit in type-A intercalated cells of *Rhcg*<sup>-/-</sup> mice. H<sup>+</sup>-ATPase staining was observed mostly in intercalated cells and was associated with the luminal membrane as well as with intracellular vesicles as described previously<sup>16</sup>. Thus, the absence of Rhcg is not associated with a redistribution of H<sup>+</sup>-ATPases in type-A intercalated cells.

### *The overexpression of RhCG in HEK293 cells increases H<sup>+</sup> secretion rates*

To test if the expression of RhCG was associated with stimulation of H<sup>+</sup>-ATPase activity, we used HEK293 cells, which express H<sup>+</sup>-ATPases and exhibit H<sup>+</sup>-ATPase-mediated H<sup>+</sup> secretion across the plasma membrane<sup>34</sup>. Non-transfected HEK293 cells and HEK293 cells stably transfected with human RhCG were used to examine the effect of RhCG expression on H<sup>+</sup>-ATPase expression and activity. qRT-PCR confirmed high expression levels of RhCG in RhCG-transfected HEK293 cells

as reported previously (Figure 6A) <sup>5</sup>. Next, we compared the expression levels of transcripts encoding different subunits of H<sup>+</sup>-ATPase, *ATP6V1B1*, *ATP6V1B2* and *ATP6V0A1*, and found an exclusive increase in mRNA expression of *ATP6V1B1* in HEK-RhCG compared to HEK-WT cells (Figure 6B-D).

Following NH<sub>4</sub>Cl prepulse acidification in a HCO<sub>3</sub><sup>-</sup> and Na<sup>+</sup> free solution, we measured intracellular pH recovery rates, similar to experiments in microperfused CCDs (Figure 7A). We have previously demonstrated that this pH<sub>i</sub> recovery is mediated solely by H<sup>+</sup>-ATPases and sensitive to the H<sup>+</sup>-ATPase blocker bafilomycin <sup>34</sup>. The initial pH<sub>i</sub> of both groups of cells measured was in the same range (pH 7.16 ± 0.001 for non-transfected HEK293 cells and pH 7.18 ± 0.002 for RhCG expressing HEK cells, Figure 7A) which allowed a direct comparison of ΔpH<sub>i</sub>/Δt. In HEK-RhCG cells, the rate of pH<sub>i</sub>-recovery after NH<sub>4</sub>Cl removal was 52 % higher than the one observed in non-transfected HEK (HEK-RhCG: (0.666 ± 0.001)\*10<sup>-3</sup> pH units/min vs. non-transfected HEK: (0.322 ± 0.006)\*10<sup>-3</sup> pH units/min, p ≤ 0.01) (Figure 7B). This result suggests that the presence of RhCG can stimulate H<sup>+</sup> secretion and is in agreement with our data showing that H<sup>+</sup> secretion is impaired in collecting ducts from *Rhcg*<sup>-/-</sup> mice.

#### *Lack of the Atp6v1b1 H<sup>+</sup>-ATPase subunit reduces H<sup>+</sup>-ATPase activity without effect on NH<sub>3</sub> permeability*

In order to assess whether H<sup>+</sup>-ATPase activity could affect Rhcg activity, we used a genetic animal model with reduced H<sup>+</sup>-ATPase activity, mice lacking the Atp6v1b1 (B1) subunit <sup>19, 35-36</sup>. *Atp6v1b1*<sup>-/-</sup> mice challenged with a HCl containing diet showed a more alkaline urinary pH compared to *Atp6v1b1*<sup>+/+</sup> mice (Figure 8A and Supplementary table 1) as demonstrated previously<sup>19</sup>. However, urinary NH<sub>4</sub><sup>+</sup> excretion was similar between both genotypes before and during acute HCl treatment. But, after 4 days of acid inducing diet, *Atp6v1b1*<sup>-/-</sup> presented with a significant 23% reduction of NH<sub>4</sub><sup>+</sup> excretion (Figure 8B). To assess whether collecting ducts from *Atp6v1b1*<sup>-/-</sup> mice had altered NH<sub>3</sub> permeability, we investigated NH<sub>3</sub> membrane permeabilities and H<sup>+</sup> secretion in ex vivo microperfused isolated CCDs from *Atp6v1b1*<sup>+/+</sup> and <sup>-/-</sup> mice receiving HCl diet for 2 days. We confirmed the expected reduction in H<sup>+</sup> secretion in *Atp6v1b1*<sup>-/-</sup> mice (Figure 8C) but did not detect any differences in NH<sub>3</sub> membrane permeability across the apical (Figure 8D) or



basolateral (Figure 89E) membranes. Thus, in this genetic model, reduced H<sup>+</sup>-ATPase activity is not paralleled by reduced NH<sub>3</sub> permeability.

## DISCUSSION

The present study provides a new and additional explanation for the well-known association between urinary acidification and ammonium excretion<sup>26</sup>. We demonstrate that the ammonia channel RhCG is required for maximal urinary acidification and that RhCG stimulates H<sup>+</sup>-ATPase activity.

Secretion of protons acidifies urine which provides a major driving force for NH<sub>3</sub> secretion and trapping of NH<sub>4</sub><sup>+</sup> after protonation. Thus, in this physico-chemical model, H<sup>+</sup>-secretion is the primary process which then affects the chemical gradient for NH<sub>3</sub> secretion.

Urinary acidification depends on H<sup>+</sup> excretion along the collecting duct mediated mostly by H<sup>+</sup>-ATPases and to a lesser extent by H<sup>+</sup>-K<sup>+</sup>-ATPases. In intercalated cells, H<sup>+</sup>-ATPases are the primary pump at the apical membrane mediating type A intercalated cell H<sup>+</sup>-excretion after an intracellular acid-load<sup>33, 37</sup>. The B1 H<sup>+</sup>-ATPase subunit is specifically represented and colocalizes with RhCG at the luminal side of renal type A intercalated cells<sup>10</sup>. Based on previous observations that *Rhcg* null mice had more alkaline urine despite reduced NH<sub>3</sub> secretion rates<sup>11-12</sup>, we hypothesized that *Rhcg* was required for maximal urinary acidification. Here, we observed a reduced apical H<sup>+</sup> secretion rate in acid-loaded *Rhcg*<sup>-/-</sup> mice suggesting a form of regulation of H<sup>+</sup>-ATPases by RhCG or its activity.

In *Rhcg*<sup>-/-</sup> mice, reduced H<sup>+</sup>-ATPase activity was not caused by down-regulation of B1 or other H<sup>+</sup>-ATPase subunits at mRNA or protein level. On the contrary, B1 was upregulated which may represent a compensatory process. The B2 H<sup>+</sup>-ATPase subunit was downregulated and as previously been shown to exhibit counterregulation in B1 deficient mice<sup>38</sup>. These data suggest that the reduction in apical H<sup>+</sup> flux observed in *Rhcg*<sup>-/-</sup> mice CDs is not the consequence of a lower amount of H<sup>+</sup>-ATPases formed but rather explained by H<sup>+</sup>-ATPases which are not fully functional due to either an inadequate assembling of the subunits or improper insertion of the pumps at the plasma membrane. Immunohistochemistry and immunogold staining for several subunits of the V<sub>1</sub> and V<sub>0</sub> domains of the H<sup>+</sup>-ATPase at the apical pole of intercalated cells did not find any evidence for altered subcellular localization and the presence of both V<sub>0</sub> and V<sub>1</sub> domain subunits suggests also intact assembly of pumps.

*In vitro* studies in HEK293 cells revealed that overexpression of RhCG stimulates H<sup>+</sup> extrusion paralleled by higher mRNA expression of the B1 H<sup>+</sup>-ATPase subunit. HEK293 cells express H<sup>+</sup>-ATPases and exhibit H<sup>+</sup> extrusion only after intracellular acidification in a slow, time dependent manner that is independent from trafficking of H<sup>+</sup>-ATPases to the plasma membrane <sup>34</sup>. Thus, H<sup>+</sup>-ATPase activity is increased in the presence of RhCG *in vivo* and *in vitro* and this effect may involve also increased abundance of some subunits, at least *in vitro*.

We next tested in *Atp6v1b1*<sup>-/-</sup> mice whether H<sup>+</sup>-ATPases would have a reciprocal regulatory effect on Rhcg activity. As previously reported, we observed higher urinary pH and highly reduced apical H<sup>+</sup> transport in acute and chronic HCl acid-loaded *Atp6v1b1*<sup>-/-</sup> mice compared to wildtype littermates <sup>19, 35-36</sup>. However, the absence of *Atp6v1b1* and reduced H<sup>+</sup>-ATPase activity did not affect the NH<sub>3</sub> transport measured on both basolateral and apical cellular membranes. Accordingly, urinary NH<sub>4</sub><sup>+</sup> excretion was not different in 2 days acid-loaded *Atp6v1b1*<sup>-/-</sup> compared to wildtype. However, only after a more chronic acid-load, urinary NH<sub>4</sub><sup>+</sup> excretion was reduced in *Atp6v1b1*<sup>-/-</sup> mice compared to *Atp6v1b1*<sup>+/+</sup> animals but to a much smaller extent than in *Rhcg*<sup>-/-</sup> mice under the same conditions <sup>11</sup>. The modest decrease in urinary NH<sub>4</sub><sup>+</sup> excretion could be due to the more alkaline urinary pH reducing the driving force for NH<sub>4</sub><sup>+</sup> secretion along the collecting system. Thus, RhCG stimulates H<sup>+</sup>-ATPase activity whereas reduced H<sup>+</sup>-ATPase activity does not directly impact on RhCG activity.

H<sup>+</sup>-ATPase activity is regulated by assembly/disassembly of the pump, trafficking to and from the membrane, and by phosphorylation at different sites which may occur in response to changes in acid-base status and/or endocrine factors <sup>3, 15, 39-51</sup>. RhCG may modulate H<sup>+</sup>-ATPase activity through at least two different mechanisms. NH<sub>3</sub> stimulates H<sup>+</sup>/K<sup>+</sup>-ATPase possibly involving increased insertion of the H<sup>+</sup>/K<sup>+</sup>-ATPase via a SNARE-dependent process <sup>52</sup>. RhCG participates in basolateral NH<sub>3</sub> uptake <sup>11</sup> and loss of RhCG activity may reduce intracellular NH<sub>3</sub> levels and NH<sub>3</sub> dependent regulation of luminal H<sup>+</sup>-extrusion. Alternatively, RhCG may stimulate H<sup>+</sup>-ATPase activity through (in)direct protein-protein interactions. The B1 H<sup>+</sup>-ATPase subunit possesses a PDZ domain to interact with other proteins <sup>46</sup>. Whether RhCG interacts with other protein has not been reported to date. However, the related RhBG protein, expressed at the basolateral side of renal CCD cells <sup>53</sup>,

has been shown to be anchored to the membrane via the actin cytoskeleton-binding protein ankyrin-G <sup>54</sup> and to be part of a structural complex where the anion exchanger 1 (AE1) responsible for the secretion of  $\text{HCO}_3^-$  into blood by the intercalated cells can bind to both RhBG and ankyrin-G <sup>55</sup>. This complex is essential for the activity of RhBG and AE1..Likewise,  $\text{H}^+$ -ATPases exhibit interactions with the actin cytoskeleton, via the C subunit <sup>56</sup> and most likely via binding sites for F-actin that are present in the amino-terminal domains of both B1 and B2 isoforms <sup>57</sup> Thus, RhCG and  $\text{H}^+$ -ATPases may be held together in one protein complex through interactions via the actin cytoskeleton. There is emerging evidence for large protein complexes involved in the regulation of  $\text{H}^+$ -ATPases as well as coordinating the development of intercalated cells. These processes are critical for the kidneys ability to maintain and adapt acid excretion. On the one hand, rare mutations may exist in some patients and their discovery will help to diagnose hitherto unexplained cases of inborn dRTA. On the other hand, dysregulation of these networks may contribute to reduce acid excretion observed in patients with more common forms of CKD. Nevertheless, a better understanding of the components of these networks is required.

In summary, we show that RhCG and  $\text{H}^+$ -ATPases are located within the same cellular protein complex in the kidney and this interaction is required for maximal urinary acidification by  $\text{H}^+$ -ATPases, a prerequisite for efficient  $\text{NH}_3$  secretion and urine excretion of  $\text{NH}_4^+$ . Thus, this novel mechanism provides a molecular explanation for the tight association between urinary acidification and ammonium excretion where RhCG mediates  $\text{NH}_3$  secretion and maximizes  $\text{H}^+$ -secretion.

## MATERIALS AND METHODS

### *Co-Immunoprecipitation*

Co-immunoprecipitation was performed using the Pierce Cross-link Magnetic beads kit (Pierce, USA) according to the manufacturers' instructions. Fifty mg of medulla from rat kidney were homogenized using an electric homogenizer in 3 ml

Pierce IP Lysis/Wash Buffer (pH 7.4, 0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% glycerol). Rabbit anti-B1 H<sup>+</sup>-ATPase<sup>33</sup> and rabbit anti-Rhcg (Proteintech, USA) polyclonal antibodies were bound to Pierce Protein A/G Magnetic Beads, and crosslinked using DSS reagent. Finally, crosslinked complexes were eluted with the pH 2.0 Pierce Elution Buffer, and neutralized with the Pierce Neutralization Buffer at pH 8.5.

*Animals* - *Rhcg*<sup>+/+</sup> and <sup>-/-</sup> mice were generated and bred as described before<sup>11</sup>. Also, *Atp6v1b1*<sup>+/+</sup> and <sup>-/-</sup> mice were bred as described before<sup>19,35</sup>. Mice were genotyped by PCR directly on a 3 µl 25 mM NaOH biopsy digestion product.

Genomic DNA was amplified using primer pairs specific for *Rhcg* exon 1: forward (AGACCCCACAATGGAAAGCTATAA) and wildtype reverse (CAACCAGAACTCCCCAGTGTCTCAGA) and knock-out reverse (ATGGGCTGACCGCTTCCTCGTGCTTTAC) and *Atp6v1b1* primer pairs specific for the *Neo* cassette: forward (CACGACGAGATCCTCGCCGTC) and reverse (GCGCAGCTAGTGCTCGACGTTG) and an *Atp6v1b1* 3' flanking sequence not included within the targeting vector: forward (CTGGCACTGACCACGGCTGAG) and reverse (CCAGCCTGTGACTGAGCCCTG). The products were separated by electrophoresis on 1% agarose gels (*Rhcg* mutant product: 522 bp, wildtype product: 376 bp, *Atp6v1b1* mutant product: 300 bp, wildtype product: 200bp).

All animal experiments were performed in acid-loading conditions for which mice were given 0.2 M HCl added to powdered standard food for 2 days. All experiments were performed according to Swiss Animal Welfare laws and were approved by the local veterinary authority (Veterinäramt Zürich).

### *In vivo experiments*

All experiments were performed using age- and sex-matched *Atp6v1b1* wildtype (*Atp6v1b1*<sup>+/+</sup>) and *Atp6v1b1* knockout (*Atp6v1b1*<sup>-/-</sup>) littermate mice (3-4

month-old), housed in metabolic cages (Techniplast, Switzerland). Mice were given deionized water ad libitum and were fed with a standard powdered laboratory chow (Kliba, Augst, Switzerland). Mice were allowed to adapt to metabolic cages for 3 days and a first retro-orbital blood sample was taken for blood gas analysis at baseline. Then, two 24 hrs urine samples were collected under light mineral oil in the urine collector to determine daily urinary parameters. Mice were then allowed to recover for 2 weeks before given a HCl-containing diet (0.2 M HCl added to powdered standard food) in normal cages. Food, water intake, and urine excretion was monitored following the same procedures as under baseline conditions. Urine collections were performed on the first, second and fourth day of acid-loading. Retro-orbital blood samples were taken on the second and fourth day of the HCl diet under light anesthesia with isoflurane. All animal experiments were conducted according to Swiss Laws of Animal Welfare and approved by the local Zurich Veterinary Authority (Kantonaes Veterinäramt Zürich).

#### *Analytic procedures*

Blood pH, pCO<sub>2</sub>, and electrolytes were measured with a pH/blood-gas analyzer (ABL77 Radiometer). Urinary pH was measured with a pHmeter (Metrohm AG, Canada) and creatinine by a modified kinetic Jaffé colorimetric method <sup>58</sup>. Urinary NH<sub>4</sub><sup>+</sup> was measured with the Berthelot protocol <sup>59</sup>. Urine inorganic phosphate (Pi) concentration was determined by the phosphomolybdate method <sup>60</sup>.

#### *Electron microscopy and immunogold labeling*

Mice were anesthetized with 2.5 % Isoflurane/pressurized air and perfused through the left ventricle with PBS (0.9% NaCl in 10 mM phosphate buffer, pH 7.4) followed by paraformaldehyde-lysine-periodate fixative (PLP: 4% paraformaldehyde, 75 mM lysine HCl, 10 mM sodium periodate, and 0.15 M sucrose in 37.5 mM sodium phosphate)<sup>61</sup>. After 5 minutes the kidneys were removed and postfixed for an additional hour on ice in the same fixative solution. Following fixation, the kidneys were stored at 4°C in PBS.

For immunogold staining, small pieces of *Rhcg*<sup>+/+</sup> and <sup>-/-</sup> kidneys were dehydrated through a graded series of ethanol to 100% ethanol and then embedded in LR White resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Thin

sections were incubated on drops of primary anti-H<sup>+</sup>-ATPase (A-subunit, 1:200) antibody for 2 h. After rinsing with PBS, the grids were incubated on drops of goat anti-rabbit IgG coupled to 15nm gold particles (Ted Pella, Redding, CA, USA) for 1 h. Following several rinses with distilled water, the grids were stained with 2% uranyl acetate for 10 min, rinsed in distilled water, and dried. Sections were examined in a JEM-1011 transmission electron microscope (JEOL Ltd, Tokyo, Japan).

### *Immunohistochemistry*

*Rhcg*<sup>+/+</sup> and <sup>-/-</sup> mice were anesthetized with ketamine/xylazine and perfused through the left ventricle with PBS followed by paraformaldehyde-lysine-periodate (PLP) fixative <sup>61</sup>. Kidneys were removed and fixed overnight at 4°C by immersion in PLP. Kidneys were washed three times with PBS, and 3 µm cryosections were cut after cryoprotection with 0.9 M sucrose in PBS for at least 1 h. Immunostaining was carried out as described <sup>62</sup>. The following primary antibodies were used: guinea pig anti-AE1 1:500 <sup>63</sup>, rabbit anti-a4 H<sup>+</sup>-ATPase 1:500 <sup>33</sup>, rabbit anti-B1 H<sup>+</sup>-ATPase <sup>33</sup>, rabbit anti-B2 H<sup>+</sup>-ATPase 1:500 <sup>64</sup>, mouse monoclonal ant E H<sup>+</sup>-ATPase 1:200 (gift from S. Gluck), rabbit anti *Rhcg* <sup>65</sup> 1:1000 and goat anti AQP-2 (Santa Cruz Biotechnology) 1:1000. The secondary antibodies were donkey anti-rabbit 594, donkey anti-goat 488, donkey anti-rabbit 488, donkey anti-mouse 594 and donkey anti-guinea pig 647 (Molecular Probes) at 1:1000.

### *Immunoblotting*

Crude total membranes were obtained from *Rhcg*<sup>+/+</sup> and <sup>-/-</sup> mouse renal medulla homogenized in 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, and in the presence of protease inhibitors (Roche Protease Inhibitor Cocktail) <sup>62</sup>. Forty micrograms of crude membrane proteins were solubilized in Laemmli loading buffer containing 10% DTT and separated on 8 to 10% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). After blocking with 5 % milk powder in Tris-buffered saline/0.1% Tween-20 for 60 min, blots were incubated with primary antibodies: rabbit anti-mouse ATP6V1B1 (B1)(1:5000) <sup>33</sup>, rabbit anti-ATP6V1B2 (B2) (1:5,000) <sup>64</sup>, rabbit anti-ATP6V0a4 <sup>33</sup>, 1:5000, rabbit anti-ATP6V0a1 (1:5000), a kind gift of Dr Xie, Dallas, TX, USA) <sup>66</sup>, and mouse anti-ATP6V1E2 (1:5000, a kind gift of Dr. S. L Gluck, University of California, San

Francisco, CA, USA)<sup>67</sup>, and mouse monoclonal anti- $\beta$ -actin antibody (Sigma, St. Louis, MO; 1:10000) overnight at 4°C. After washing and blocking with 5 % milk powder for 60 min, membranes were incubated for 2 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies 1:5000 linked to alkaline phosphatase (Promega, Madison, WI, USA). The protein signal was detected with the appropriate substrate (Millipore Corp, Bedford, MA, USA) using the las-4000 image analyzer system (Fujifilm Life Science USA). All images were analyzed using Advanced Image Data Analyzer AIDA (Raytest, Straubenhardt, Germany) to calculate the protein of interest/ $\beta$ -actin ratio.

### *Cell culture*

Non-transfected HEK293 (human embryonic kidney 293) or stably transfected with human RhCG (HEK-RhCG) cell lines were generated as previously described<sup>6</sup> and grown at 37°C, 5% CO<sub>2</sub> in DMEM (Dulbecco's modified Eagle's medium)/Glutamax I (Invitrogen) supplemented with 10% (v/v) fetal bovine serum. Selection of HEK-RhCG cells was performed in culture medium supplemented with 0.3 mg/ml hygromycin (Invitrogen). For all pH experiments, cells were passaged, disseminated onto coverslips and grown to sub-confluency for 48 hours. The final medium exchange was performed 24 hours before the experiments were started. Medium pH was maintained at pH 7.4.

### *RNA extraction and reverse transcription*

Snap-frozen kidneys (five kidneys for each condition) or non-transfected HEK and HEK-RhCG cells (3 petri dishes of 5x10<sup>6</sup> cells for each condition) were homogenized in RLT-Buffer (Qiagen, Basel, Switzerland) supplemented with  $\beta$ -mercaptoethanol to a final concentration of 1%. Total RNA was extracted from 200  $\mu$ l aliquots of each homogenized sample using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Quality and concentration of the isolated RNA preparations were analyzed on the ND-1000 spectrophotometer (Nano-Drop Technologies). Total RNA samples were stored at -80°C. Each RNA sample was diluted to 100 ng/ $\mu$ l and 3  $\mu$ l used as a template for reverse transcription using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For reverse transcription, 300 ng of RNA template were diluted in a 40  $\mu$ l reaction mix that contained (final concentrations) RT buffer (1 x), MgCl<sub>2</sub> (5.5 mM),



random hexamers (2.5  $\mu$ M), RNase inhibitor (0.4U/ $\mu$ l), the multiscribe reverse transcriptase enzyme (1.25U/ $\mu$ l), dNTP mix (500  $\mu$ M each), and RNase-free water.

#### *Reverse Transcription and semiquantitative real-time PCR*

Semiquantitative real-time RT-qPCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Forster City, CA). Primers and probes were chosen using BLAST tool: Ensemble (<http://www.ensembl.org/index.html>) to result in amplicons no longer than 150 bp spanning intron-exon boundaries to exclude genomic DNA contamination. The specificity of all primers was first tested on mRNA derived from kidney and always resulted in a single product of the expected size (data not shown). Probes were labeled with the reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth, Balgach, Switzerland). Primers and probes sequences are summarized in supplementary table 3.

Real-Time PCR reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Forster City, CA). Briefly, 3  $\mu$ l cDNA, 0.8  $\mu$ l of each primer (25  $\mu$ M), 0.4  $\mu$ l labeled probe (5  $\mu$ M), 5  $\mu$ l RNase-free water, and 10  $\mu$ l TaqMan Universal PCR Master Mix reached 20  $\mu$ l of final reaction volume. Reaction conditions were denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s and annealing/elongation at 60°C for 60s with auto ramp time. All reactions were run in triplicate. For analyzing the data, the threshold was set to 0.2 as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of the genes of interest was calculated in relation to hypoxanthine guanine phosphoribosyl transferase (mouse HPRT or human HPRT). Relative expression ratios were calculated as  $R = 2^{(Ct(HPRT) - Ct(test\ gene))}$ , where Ct represents the cycle number at the threshold 0.02.

#### *Intracellular pH (pH<sub>i</sub>) measurements on HEK293 cells*

For pH<sub>i</sub> measurements, individual slides seeded with non-transfected HEK or HEK-RhCG cells were transferred into a perfusion chamber (~ 3-5 ml/min flow rate) and mounted on an inverted microscope (Zeiss Axiovert 200, Carl Zeiss, Feldbach, Switzerland) equipped with a video imaging system (Visitron, Munich, Germany) for the duration of the experiment. The temperature of the chamber was maintained at 37°C by an electronic feedback circuit. The control bath solution was initially a

HEPES-buffered Ringer solution (125 mM NaCl/ 5 mM KCl/ 1 mM  $\text{CaCl}_2$ / 1.2 mM  $\text{MgSO}_4$ / 2 mM  $\text{NaH}_2\text{PO}_4$ / 32.2 mM HEPES/ 5 mM Glucose). Cells were loaded with the acetoxymethyl ester of the pH sensitive dye BCECF (10  $\mu\text{M}$ , 2',7'-bis(2-carboxyl)-5-(and-6)-carboxyfluorescein, Invitrogen, Switzerland) for 15 min and washed to remove all non-deesterified dye.  $\text{pH}_i$  was measured by microfluorometry by exciting the BCECF dye with a spot of light alternately at 490 and 440 nm while monitoring the emission at 532 nm with a video-imaging system <sup>68</sup>.  $\text{H}^+$ -ATPase activity was measured after induction of a sharp intracellular acidification using the  $\text{NH}_4\text{Cl}$  (20 mM) prepulse technique <sup>32, 68</sup>. Bicarbonate-free solutions were used and  $\text{Na}^+$  was replaced by N-methyl-D-glucamine (NMDG). Each experiment was calibrated for  $\text{pH}_i$  using the nigericin/high  $\text{K}^+$  method <sup>69</sup> and the obtained ratios were converted to  $\text{pH}_i$ .  $\text{Na}^+$ -independent  $\text{pH}_i$  recovery rates in response to an acid load were calculated in non-transfected HEK293 and HEK293 stably transfected cells with RhCG. Data are shown as changes in  $\text{pH}_i$  ( $\Delta\text{pH}_i$ ) per minute.

#### *Microperfusion of isolated cortical collecting ducts*

Male *Rhcg*<sup>+/+</sup>, *Rhcg*<sup>-/-</sup>, *Atp6v1b1*<sup>+/+</sup>, and *Atp6v1b1*<sup>-/-</sup> mice were given a HCl-containing diet (a 50:75 mixture (w/V) of normal powdered chow, Kliba Nafag and 0.33 M HCl) in normal cages for 2 days. Mice were anesthetized with Xylazin/Ketamin i.p. Both kidneys were cooled *in situ* with control bath solution (see below) for 1 min and then removed and cut into thin coronal slices for tubule dissection. Cortical CDs (CCDs) were dissected from the cortex at 10°C in the control solution under a stereo-microscope.

#### *Intracellular $\text{pH}_i$ measurement in microperfused cortical collecting ducts*

The isolated tubules were transferred to the bath chamber on the stage of an inverted microscope (IX81, Olympus, Japan) in the control solution containing (in mM) 138 NaCl, 1.5  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 2  $\text{K}_2\text{HPO}_4$ , 10 HEPES, 5.5 glucose, 5 alanine, pH 7.40) and then mounted on concentric pipettes and perfused *in vitro* with  $\text{Na}^+$ -free, ammonium-free solution where N-methyl-D-Glutamine<sup>+</sup> (NMDG<sup>+</sup>) replaced  $\text{Na}^+$ . The average tubule length exposed to bath fluid was limited to 300 – 350  $\mu\text{m}$  in order to prevent motion of the tubule. CCDs were loaded with 5  $\mu\text{M}$  of the fluorescent probe BCECF for ~20 min at 37°C in the control bath solution. Intracellular pH ( $\text{pH}_i$ ) recordings were made over regions of interest comprising of several cells, however,

the signal came mostly from intercalated cells that had taken up more BCECF-AM than principal cells. The loading solution was then washed out by initiation of bath flow and the tubule was equilibrated with dye-free control bath solution for 5 min. Bath solution was delivered at a rate of 20 ml/min and warmed to 37°C by water jacket immediately upstream to the chamber. After this temperature equilibration in control solution, tubules were first transiently acidified by peritubular Na<sup>+</sup> removal (Na-free, ammonium-free solution) (10 min duration), replaced by NMDG<sup>+</sup> to avoid exit of NH<sub>4</sub><sup>+</sup> by basolateral Na<sup>+</sup>-coupled transport. This maneuver was performed in the luminal absence of Na<sup>+</sup>. During the fluorescence recording, perfusion solution was delivered to the perfusion pipette via a chamber under an inert gas (N<sub>2</sub>) pressure (around 1 bar) connected through a manual 6-way valve. With this system, opening of the valve instantaneously activates flow of solutions. The majority of the fluid delivery to the pipette exits the rear of the pipette system through a drain port at 4 ml/min. This method results in a smooth and complete exchange of the luminal or the peritubular solution in less than 3 to 4 s<sup>70</sup>. After the fluorescence signal stabilization, luminal medium was instantly (at the rate of 4 ml/min in the draining) replaced by a Na<sup>+</sup>-free solution containing 20 mM NH<sub>4</sub>Cl (and 118 mM NMDG-Cl) that elicited a rapid intracellular alkalinization, followed by a sharp acidification. The rate of intracellular alkalinization has been associated with the entry of NH<sub>3</sub> whereas the subsequent phase of intracellular acidification in the continued presence of extracellular NH<sub>4</sub>Cl reflects mostly NH<sub>4</sub><sup>+</sup> entry<sup>12, 32</sup>. After the acidification reached a plateau, NH<sub>4</sub>Cl was removed from the lumen to further acidify cells and initiate direct H<sup>+</sup>-excretion with a maximum rate. All measurements were done at maximum rate for the determination of H<sup>+</sup>-ATPase activity. The measured light intensities were digitized with the CellM&CellR Imaging hardware system (Olympus, Japan) for further analysis as previously described<sup>11-12, 53</sup>. For peritubular ammonium pulses, the peritubular solution was changed to a 6 mM NH<sub>4</sub>Cl solution, pH 7.40 in the presence of 1 mM furosemide and 2.5 mM ouabain in the bath.

#### *Intrinsic buffering capacity determination*

The intrinsic buffering capacity (β<sub>i</sub>) of CCD cells was determined for the *Rhcg* strain, as previously reported<sup>11, 71</sup>, using a 40 mM NH<sub>4</sub>Cl basolateral pulse to acidify cells. To exclude HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> as a buffering component and block Na<sup>+</sup>-dependent pH<sub>i</sub> regulatory mechanisms, Na<sup>+</sup>-free, HEPES-buffered solutions were used in perfusate

and bath containing 1 mM amiloride and bath and perfusate also contained 100  $\mu$ M Sch28080 and 200 nM Concanamycin A. Addition of 40 mM  $\text{NH}_4\text{Cl}$  to the bath induced an increase followed by a decrease in  $\text{pH}_i$ . Cellular buffering power ( $\beta_i$ ) was measured in CCDs from *Rhcg*<sup>+/+</sup> and *Rhcg*<sup>-/-</sup> mice.

### *Statistics*

Statistical comparisons were tested by unpaired t-test using Graphpad Prism (GraphPad Software). P values < 0.05 were considered as statistically significant.

### **DISCLOSURE**

All the authors declared no competing interests.

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## FIGURE LEGENDS

### FIGURE 1. The B1 and B2 H<sup>+</sup>-ATPase subunits and Rhcg coexist in the same protein complex

Co-Immunoprecipitation was performed on rat kidney medulla using crosslinking. **Panel A:** Co-IP was performed with anti-Rhcg (lane 2) and anti-B1 (lane 5) polyclonal antibodies or with an isotypic IgG antibody (lanes 1 and 4). Lanes 3 and 6 were loaded with 50 mg rat kidney medulla protein. Immunoblotting (IB) was performed against the B1 H<sup>+</sup>-ATPase subunit (left side) or against RhCG (right side). **Panel B:** the Co-IP membrane was stripped and reblotted with antibodies against Rhcg and B1 H<sup>+</sup>-ATPase antibodies as control for the IP. The upper band for B1 (approx 70 kDa) is considered unspecific. **Panel C:** Co-IP was performed with anti-Rhcg (lane 2) and anti-B2 (lane 5) polyclonal antibodies or with an isotypic IgG antibody (lanes 1 and 4). Lanes 3 and 6 were loaded with 50 mg rat kidney medulla protein. Immunoblotting (IB) was performed against the B2 H<sup>+</sup>-ATPase subunit (left side) or against RhCG (right side). **Panel D:** the Co-IP membrane was stripped and reblotted with antibodies against Rhcg and B2 H<sup>+</sup>-ATPase antibodies as control for the IP. **Panel E:** Coimmunostaining of Rhcg and the E H<sup>+</sup>-ATPase subunit in kidney tissue from Rhcg<sup>+/+</sup> and Rhcg<sup>-/-</sup> mice. Immunostaining of E H<sup>+</sup>-ATPase subunit (in red) and Rhcg (green) was performed on kidney sections from Rhcg<sup>+/+</sup> and Rhcg<sup>-/-</sup> mice. Nucleus were labelled with DAPI staining. Inserts show higher magnification of single intercalated cells.

### FIGURE 2. H<sup>+</sup> secretion is reduced in cortical collecting duct cells from Rhcg<sup>-/-</sup> mice.

Cortical collecting ducts were isolated from kidneys of Rhcg<sup>+/+</sup> and Rhcg<sup>-/-</sup> mice after 2 days of HCl-loading, microperfused *in vitro*, and intracellular pH (pH<sub>i</sub>) monitored. NH<sub>4</sub>Cl (20 mM) was applied from the luminal perfusate. **(A)** Original pH<sub>i</sub> tracing from a CCD exposed to a luminal NH<sub>4</sub>Cl pulse. The arrow indicates the rate of pH<sub>i</sub> change measured and calculated. Exposure to an NH<sub>4</sub>Cl pulse caused a large intracellular acidification (not shown) followed by an alkalinization phase leading to pH<sub>i</sub> recovery. The initial slope ( $\Delta\text{pH}_i/\Delta t$ ) of the alkalinization phase was measured. **(B)** Bar graph

summarizing  $\Delta\text{pH}_i/\Delta t$  after removal of the luminal  $\text{NH}_4\text{Cl}$  pulse ( $n = 8-12$  tubules/genotype). **(C)** Bar graph summarizing  $\text{H}^+$ -fluxes calculated based on intracellular buffering power and  $\text{pH}_i$  recovery rates.

**FIGURE 3. Increased B1 and decreased B2  $\text{H}^+$ -ATPase subunit abundance in  $\text{Rhcg}^{-/-}$  mice.**

Protein expression of the B1 **(A)**, B2 **(B)**, E2 **(C)**,  $\alpha 1$  **(D)** and  $\alpha 4$  **(E)** subunits of the  $\text{H}^+$ -ATPase was assessed by Western blotting in medullary kidney tissue. In  $\text{Rhcg}^{-/-}$  mice, B1- $\text{H}^+$ -ATPase protein abundance was upregulated **(A)**, while B2  $\text{H}^+$ -ATPase was downregulated **(B)**. Protein expression of the  $\alpha 1$ ,  $\alpha 4$ , and E subunits remained unchanged **(C-E)**. Values are mean  $\pm$  SEM ( $n = 5$  mice), \*\*\* $p \leq 0.001$ .

**FIGURE 4. Unchanged  $\text{H}^+$ -ATPase subunit localization in  $\text{Rhcg}^{-/-}$  type A intercalated cells.**

Immunostaining of B1, B2 and  $\alpha 4$  subunits (in red) of three different  $\text{H}^+$ -ATPase subunits (B1, B2, and  $\alpha 4$ ) was performed on kidney sections from  $\text{Rhcg}^{+/+}$  and  $\text{Rhcg}^{-/-}$  mice. Type A intercalated cells were identified by the presence of the anion exchanger AE1 (green) and principal cells using the apical water channel AQP2 (in blue). Inserts show higher magnification of single intercalated cells.

**FIGURE 5. Subcellular localization of  $\text{H}^+$ -ATPases in type A intercalated cells is similar in  $\text{Rhcg}^{+/+}$  and  $\text{Rhcg}^{-/-}$  mice.**

Immunogold staining for the A subunit of the  $\text{H}^+$ -ATPase revealed a similar density of  $\text{H}^+$ -ATPases at the apical membrane and in apically localized vesicles of type A intercalated cells in  $\text{Rhcg}^{+/+}$  (upper panels) and  $\text{Rhcg}^{-/-}$  (lower panels) mice. Magnification of the boxed areas of the overview electron micrograph are shown on the right.

**FIGURE 6. Overexpression of RhCG in HEK293 cells stimulates ATP6V1B1 expression.**

**(A)** HEK293 cells were left untransfected (HEK293-WT) or transfected to stably express RhCG (HEK293-RhCG) and RhCG expression was confirmed using RT-qPCR. mRNA expression of ATP6V1B1 **(B)**, ATP6V1B2 **(C)** and ATP6V0A1 **(D)** were

measured by RT-qPCR in HEK293-WT and RhCG overexpressing cells HEK293-RhCG. ATP6V1B1 mRNA was higher in HEK293-RhCG cells versus HEK293-WT. The mRNA abundance of the other H<sup>+</sup>-ATPase subunits, ATP6V1B2 and ATP6V0A1, were unchanged. Values are mean  $\pm$  SEM (n= 5 dishes), \*p  $\leq$  0.05, \*\*\*  $\leq$  0.001

**FIGURE 7. RhCG overexpression in renal HEK293 cells increases H<sup>+</sup> excretion.**

H<sup>+</sup>-ATPase activity was measured in non-transfected HEK293 cells (HEK293-WT) and HEK293 cells stably transfected with human RhCG (HEK293-RhCG) using the NH<sub>4</sub>Cl (20 mM) prepulse after loading cells with BCECF. **(A)** Original pH<sub>i</sub> tracings from WT) and RhCG overexpressing HEK293 cells. Dashed lines indicate the slope measured to calculate pH<sub>i</sub> changes over time. **(B)** Bar graph summarizes the H<sup>+</sup> extrusion rate of HEK-WT compared to HEK-RhCG after NH<sub>4</sub>Cl pulse (n = 5 cells/plate, 3 dishes/condition). Data are mean  $\pm$  SEM, \*\*p  $\leq$  0.01,

**FIGURE 8. Atp6v1b1<sup>-/-</sup> mice lacking the B1 H<sup>+</sup>-ATPase subunit have decreased H<sup>+</sup>-ATPase activity but preserved apical and basolateral NH<sub>3</sub> permeabilities.**

Wildtype (Atp6v1b1<sup>+/+</sup>) and Atp6v1b1<sup>-/-</sup> mice were observed in metabolic cages and urinary pH **(A)** and urinary ammonium excretion normalized to creatinine **(B)** monitored during baseline and an acid-load (HCl) for 4 days (n = 7/genotype). Cortical collecting ducts from mice acid-loaded for 2 days were microperfused in vitro and H<sup>+</sup> secretion **(C)** and apical NH<sub>3</sub> permeability **(D)** measured after a luminal NH<sub>4</sub>Cl prepulse (20 mM) (n = 10 tubules/genotype). **(E)** Similarly, basolateral NH<sub>3</sub> permeability assessed after an NH<sub>4</sub>Cl prepulse from the bath (6 mM) was not different between genotypes (n = 6 tubules/genotype). \*p  $\leq$  0.05

## SUPPLEMENTS

**Supplementary table 1. Blood values in C57Bl/6 mice and Atp6v1b1<sup>-/-</sup> mice under normal diet and HCl load for 4 days.** Data are presented as mean  $\pm$  SEM, n = 5-9 animals/ group, \* p<0.05 vs baseline period in same genotype, # p<0.05 vs C57BL6 mice during the same period.

**Supplementary table 2. Body weight, food intake, and urinary values of C57BL6 mice and Atp6v1b1<sup>-/-</sup> mice under normal diet and during an HCl load for 4 days.** Data are presented as mean  $\pm$  SEM, n = 5-9 animals/ group, \* p<0.05 vs baseline period in same genotype, # p<0.05 vs C57BL6 mice during the same period.

**Supplementary table 3. Sequences of the primers and probes used for RT-qPCR**

**Supplementary figure 1. Unchanged mRNA expression of the B1, B2 and a4 subunits of the H<sup>+</sup>-ATPase in Rhcg<sup>+/+</sup> and Rhcg<sup>-/-</sup> mice.**

RT-qPCR was performed on total kidney to determine relative mRNA levels of several H<sup>+</sup>-ATPase relevant for intercalated cell H<sup>+</sup>-ATPase function. None of the mRNA values measured of the B1 (**A**), B2 (**B**) and **a4** (**C**) isoforms was different between the 2 genotypes. Values are mean  $\pm$  SEM (n= 5 mice).



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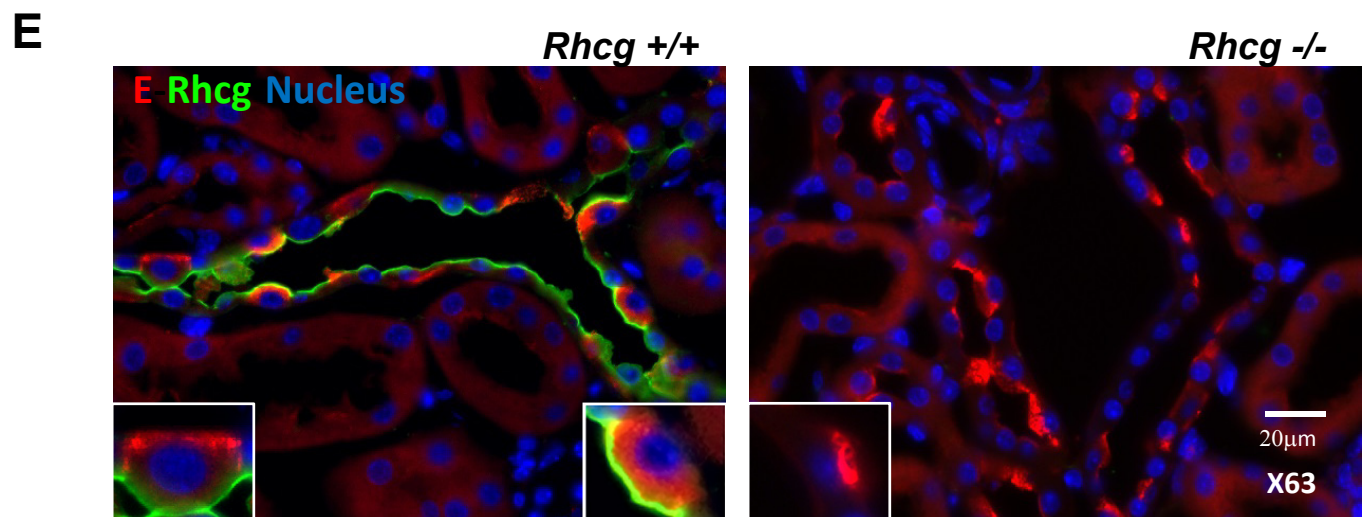
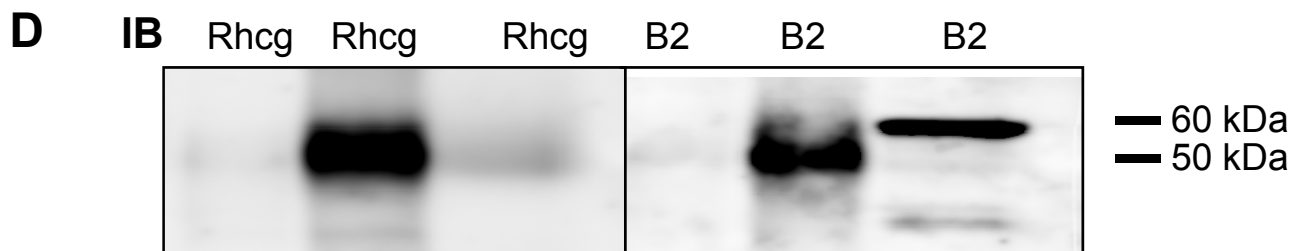
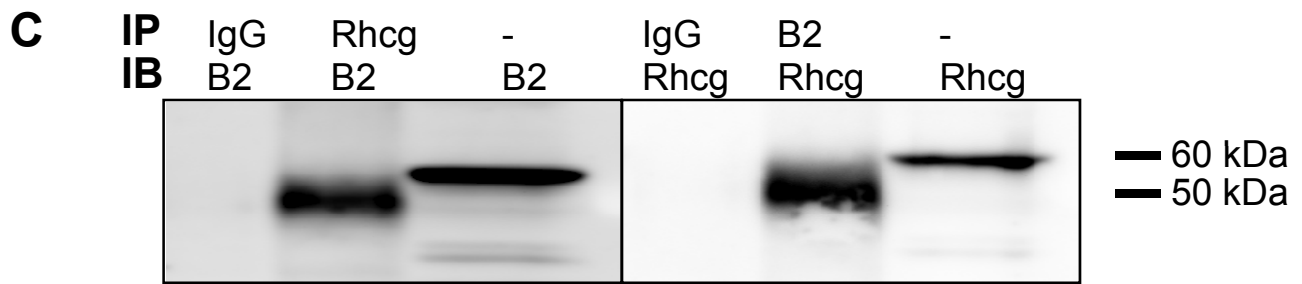
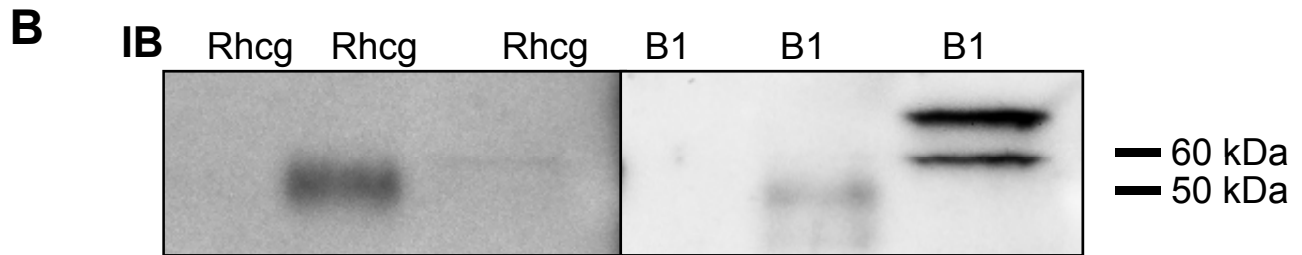
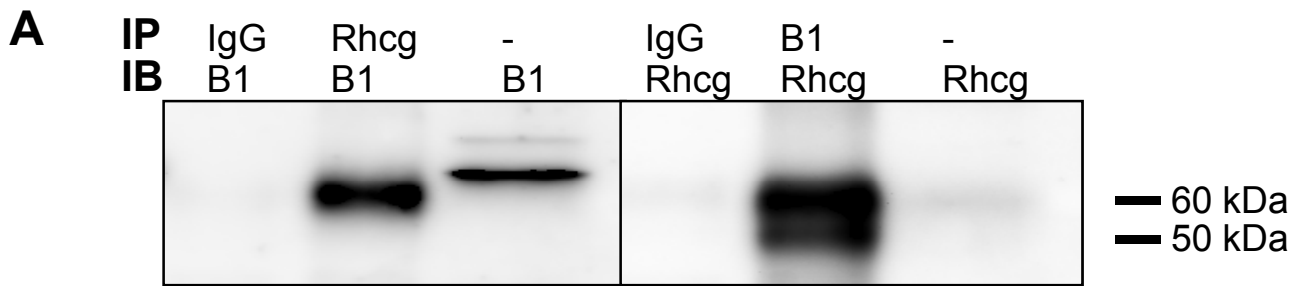
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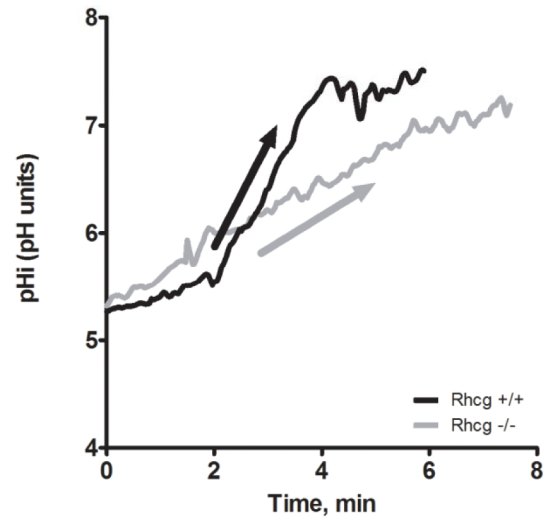
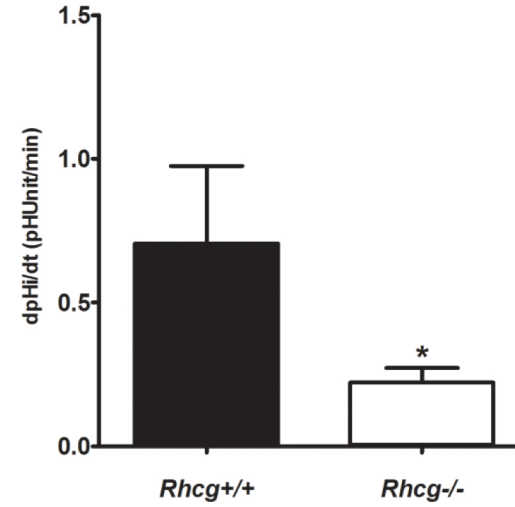
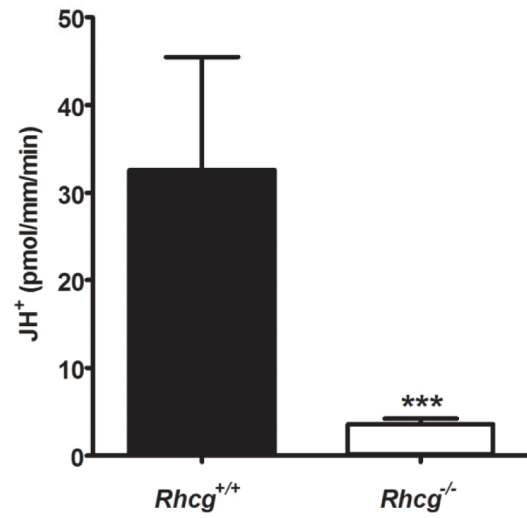
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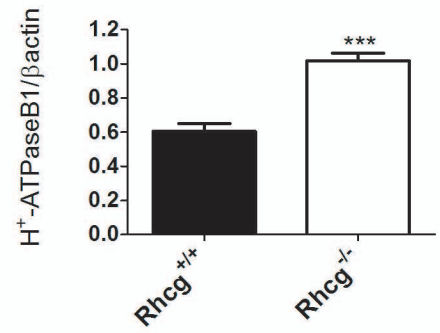
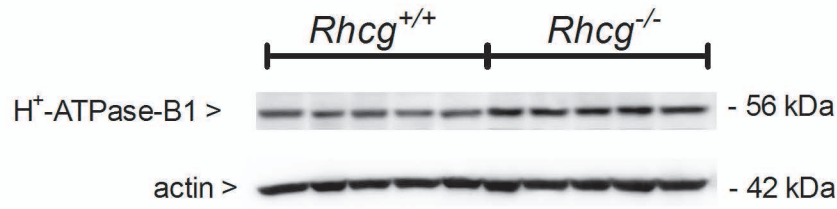
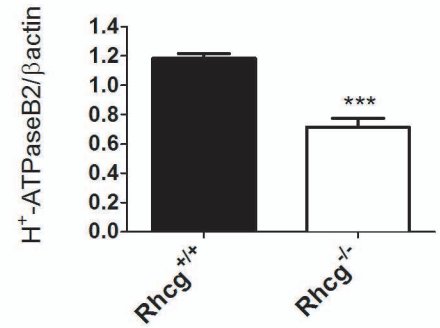
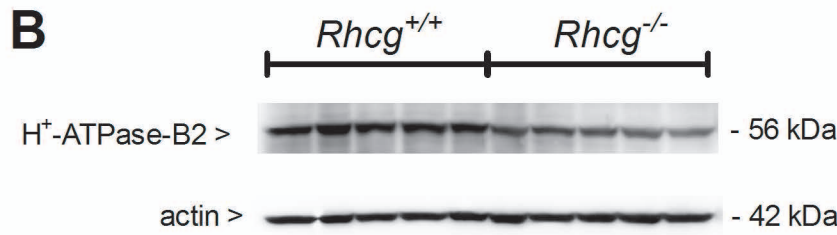
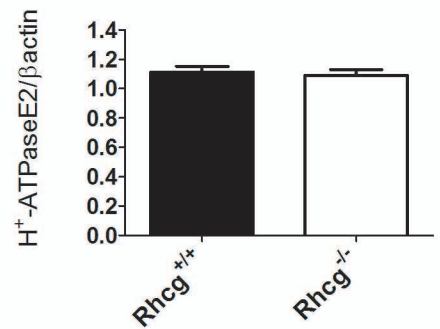
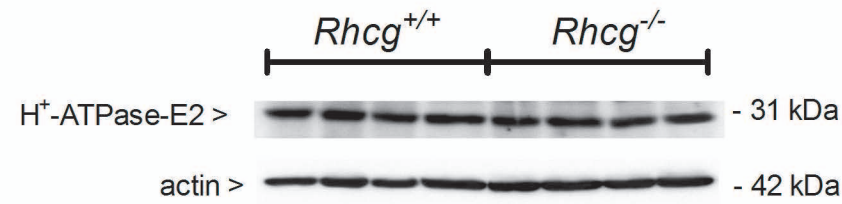
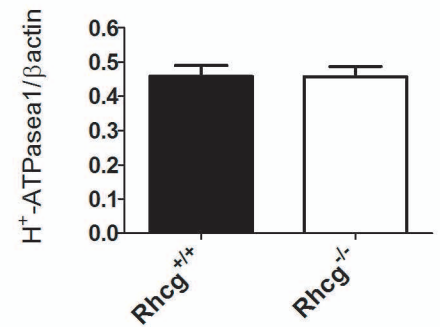
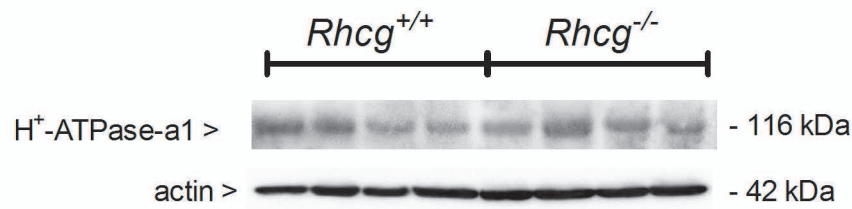
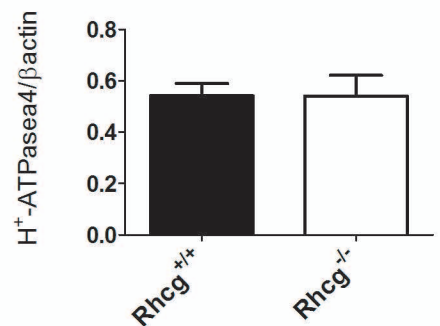
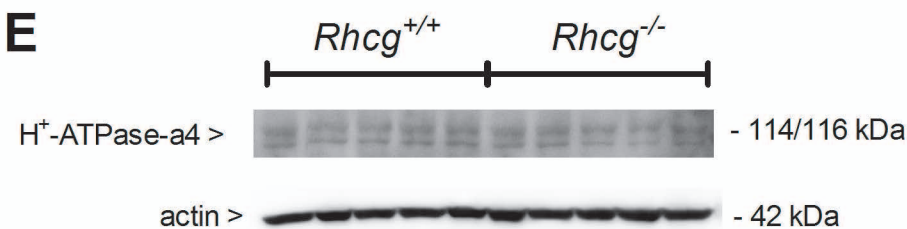
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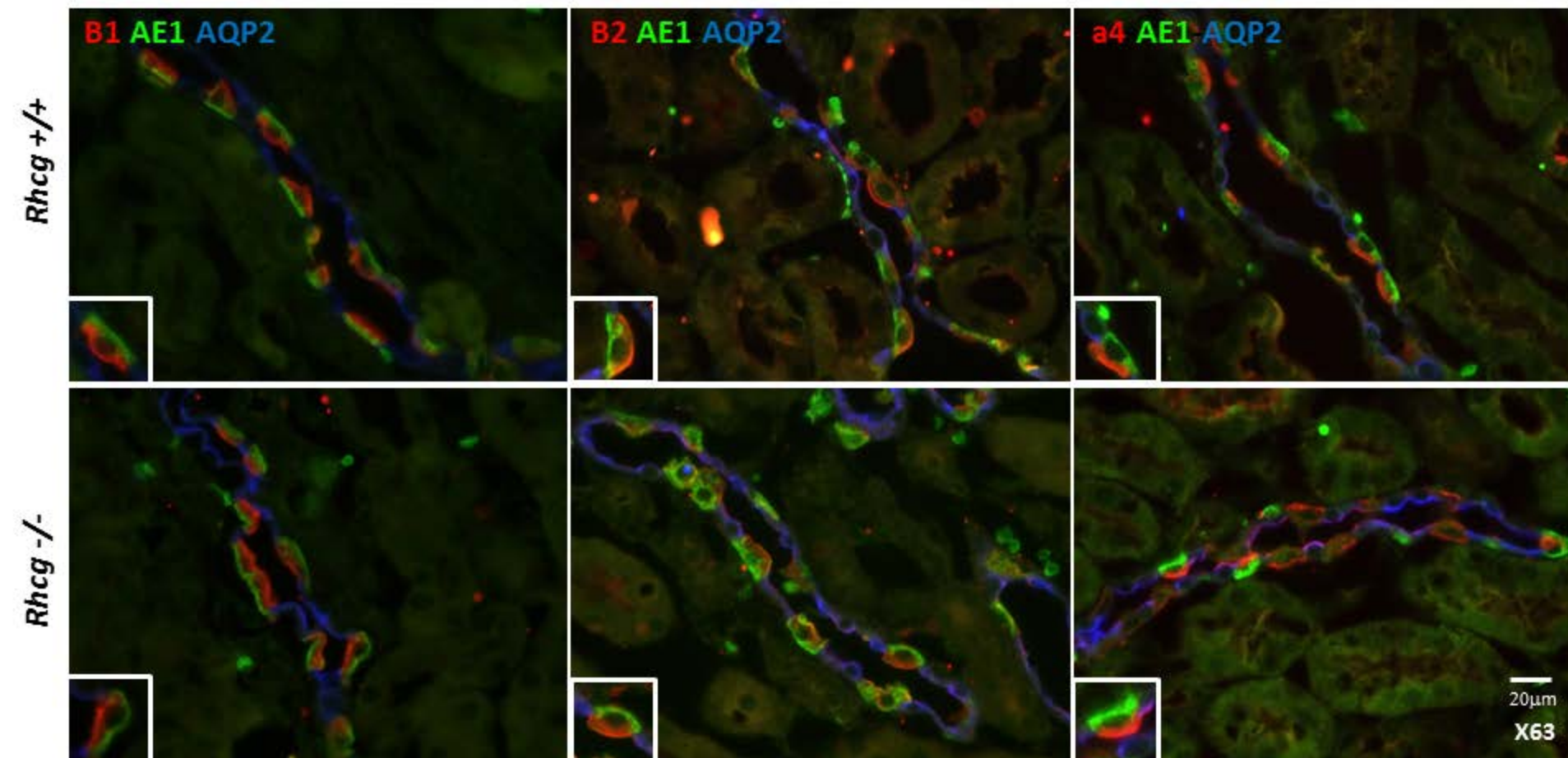
**Bourgeois, Bounoure et al., Fig 1**



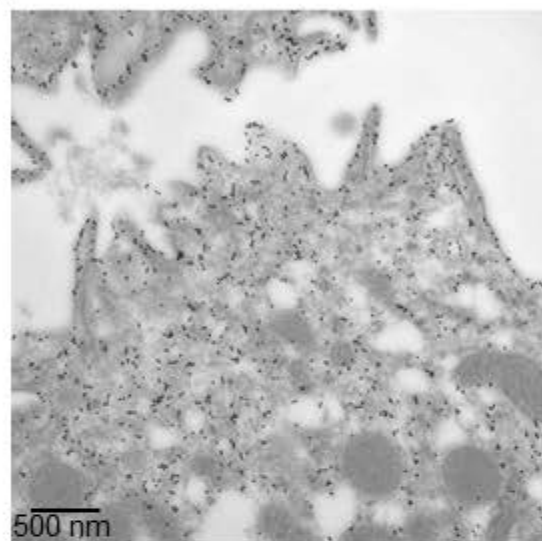
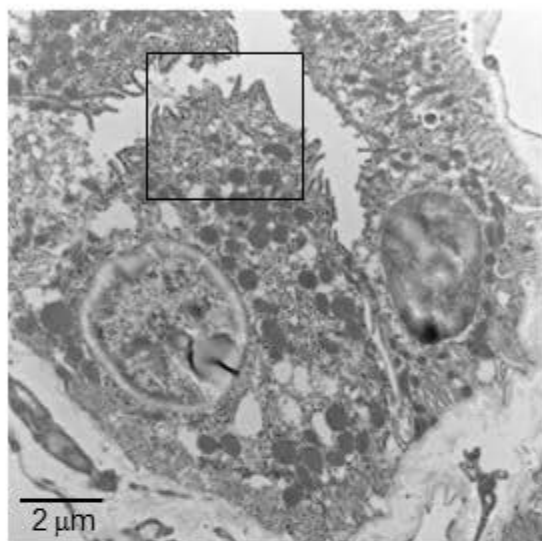


**A****B****C**

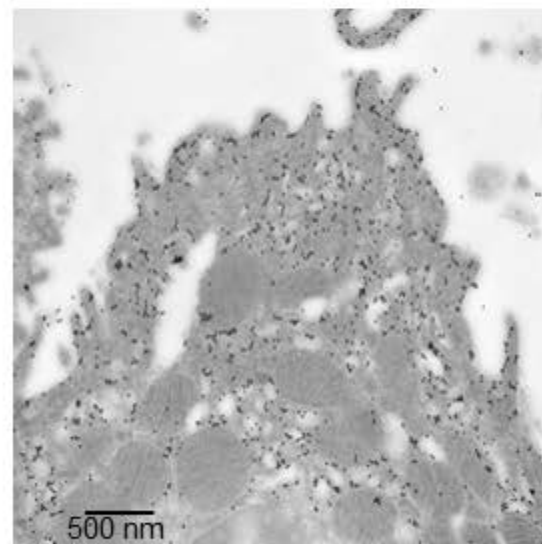
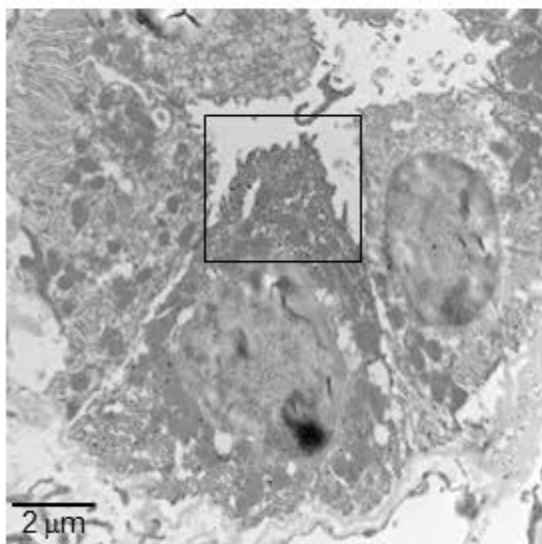
**A****B****C****D****E**

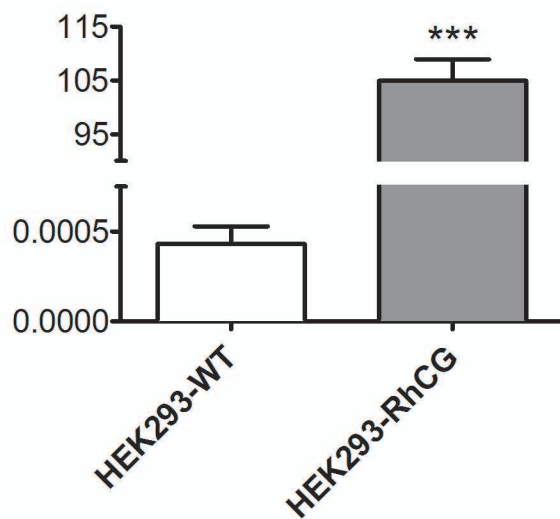
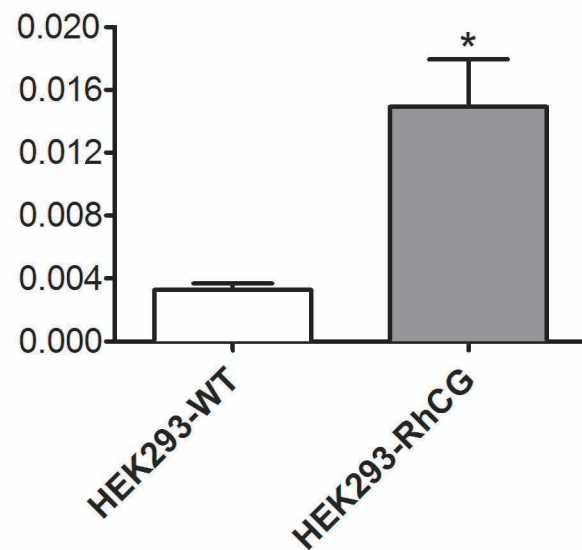
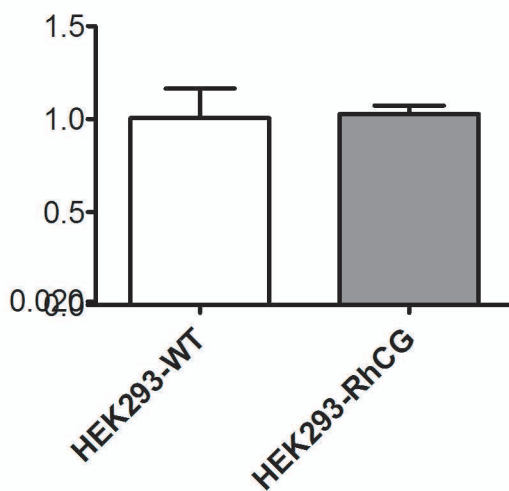
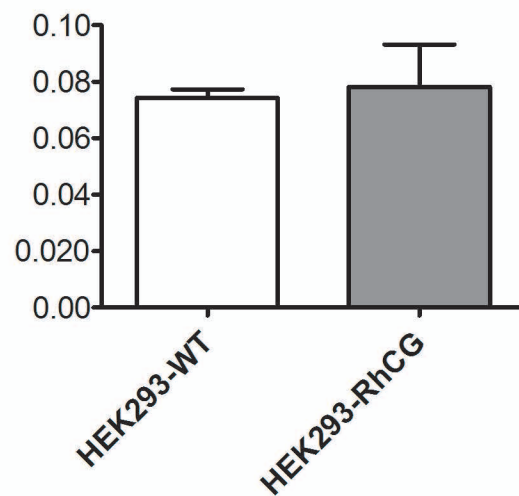


***Rhcg*<sup>+/+</sup>**

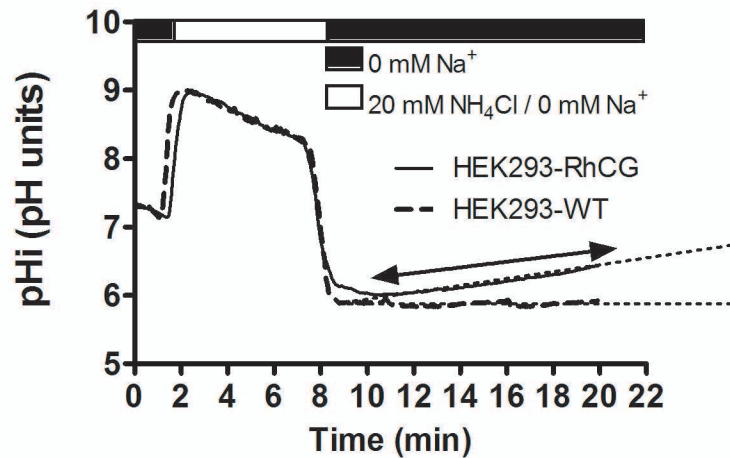


***Rhcg*<sup>-/-</sup>**

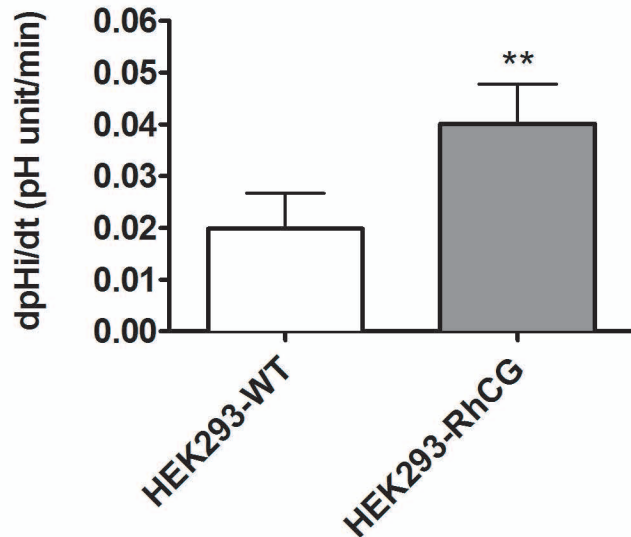


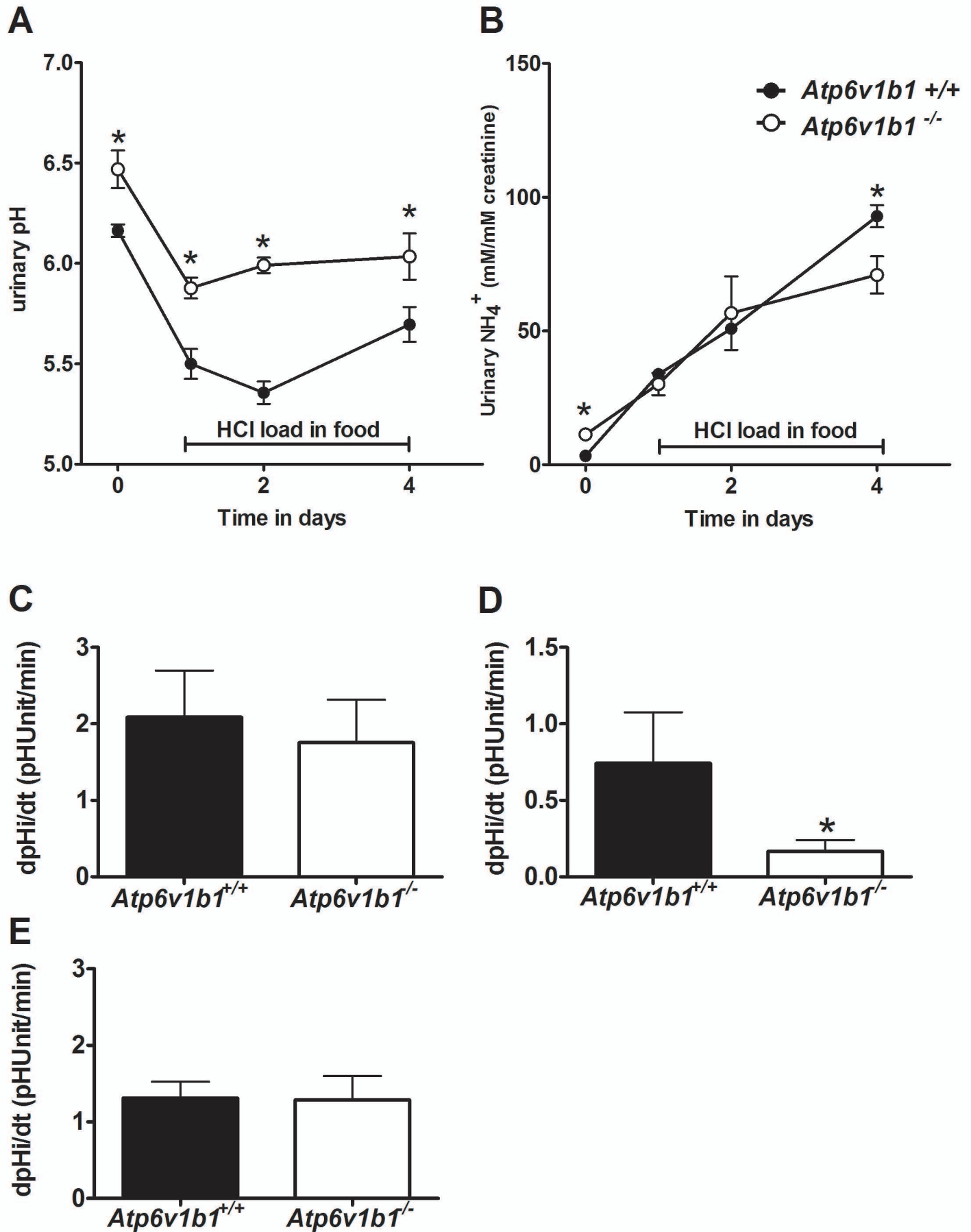
**A** $2(\text{CtHPRT}-\text{CtRhCG})$ **B** $2(\text{CtHPRT}-\text{CtATP6V1B1})$ **C** $2(\text{CtHPRT}-\text{CtATP6V1B2})$ **D** $2(\text{CtHPRT}-\text{CtATP6V0A1})$ 

**A**



**B**







## SUPPLEMENTS

**Supplementary table 1. Blood values in C57Bl/6 mice and Atp6v1b1<sup>-/-</sup> mice under normal diet and HCl load for 4 days.** Data are presented as mean  $\pm$  SEM, n = 5-9 animals/ group, \* p<0.05 vs baseline period in same genotype, # p<0.05 vs C57BL6 mice during the same period.

**Supplementary table 2. Body weight, food intake, and urinary values of C57BL6 mice and Atp6v1b1<sup>-/-</sup> mice under normal diet and during an HCl load for 4 days.** Data are presented as mean  $\pm$  SEM, n = 5-9 animals/ group, \* p<0.05 vs baseline period in same genotype, # p<0.05 vs C57BL6 mice during the same period.

**Supplementary table 3. Sequences of the primers and probes used for RT-qPCR**

**Supplementary figure 1. Unchanged mRNA expression of the B1, B2 and a4 subunits of the H<sup>+</sup>-ATPase in Rhcg<sup>+/+</sup> and Rhcg<sup>-/-</sup> mice.**

RT-qPCR was performed on total kidney to determine relative mRNA levels of several H<sup>+</sup>-ATPase relevant for intercalated cell H<sup>+</sup>-ATPase function. None of the mRNA values measured of the B1 (**A**), B2 (**B**) and **a4** (**C**) isoforms were different between the 2 genotypes. Values are mean  $\pm$  SEM (n= 5 mice).



## Supplementary data

**Supplementary table 1. Blood values in *Atp6v1b1*<sup>+/+</sup> mice and *Atp6v1b1*<sup>-/-</sup> mice under normal diet and HCl load for 4 days**

	Basal status		2 days HCl		4 days HCl	
	<i>C57BL6</i> (n=9)	<i>Atp6v1b1</i> <sup>-/-</sup> (n=7)	<i>C57BL6</i> (n=7)	<i>Atp6v1b1</i> <sup>-/-</sup> (n=6)	<i>C57BL6</i> (n=7)	<i>Atp6v1b1</i> <sup>-/-</sup> (n=5)
pH	7.35 ± 0.01	7.40 ± 0.01	7.11 ± 0.02*	7.15 ± 0.02*	7.24 ± 0.02	6.87 ± 0.12* <sup>#</sup>
pCO <sub>2</sub> (mmHg)	40.0 ± 0.6	40.6 ± 1.2	37.3 ± 1.2	39.3 ± 1.5	39.4 ± 1.5	45.4 ± 2.5 <sup>#</sup>
HCO <sub>3</sub> (mM)	21.6 ± 0.7	24.8 ± 1.2	11.5 ± 0.8*	12.8 ± 0.6*	16.4 ± 0.9*	8.7 ± 1.8* <sup>#</sup>
pO <sub>2</sub>	52.1 ± 2.2	48.8 ± 2.0	60.4 ± 2.4	62.6 ± 3.4	58.4 ± 2.5	83.8 ± 8.7* <sup>#</sup>
Na <sup>+</sup> (mM)	144.6 ± 0.5	145.4 ± 0.3	148.0 ± 1.1*	147.8 ± 1.5	151.4 ± 0.5*	150.8 ± 2.4*
Cl <sup>-</sup> (mM)	109.7 ± 0.6	108.3 ± 0.7	123.4 ± 1.6*	121.0 ± 1.5*	120.0 ± 1.6*	133.6 ± 2.8* <sup>#</sup>
Ca <sup>2+</sup> (mM)	1.26 ± 0.01	1.23 ± 0.01	1.34 ± 0.01*	1.33 ± 0.02*	1.36 ± 0.02*	1.60 ± 0.1* <sup>#</sup>
Hb (g/dl)	15.1 ± 0.2	15.7 ± 0.2	15.6 ± 0.3	16.7 ± 0.2* <sup>#</sup>	15.3 ± 0.2	18.8 ± 1.2* <sup>#</sup>

\* p<0.05 vs baseline period in same genotype, # p<0.05 vs *Atp6v1b1*<sup>+/+</sup> mice during the same period.

**Supplementary table 2. Weight, food intake and urinary values in *Atp6v1b1*<sup>+/+</sup> mice and *Atp6v1b1*<sup>-/-</sup> mice under normal diet and during an HCl load for 4 days**

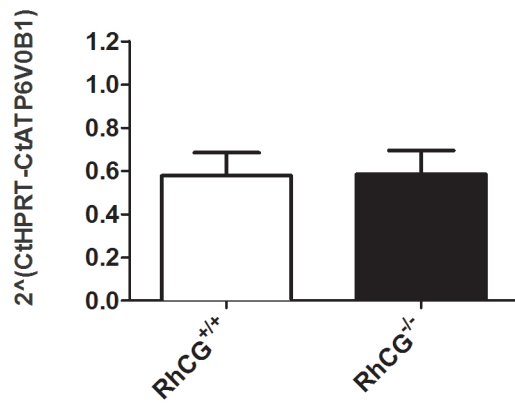
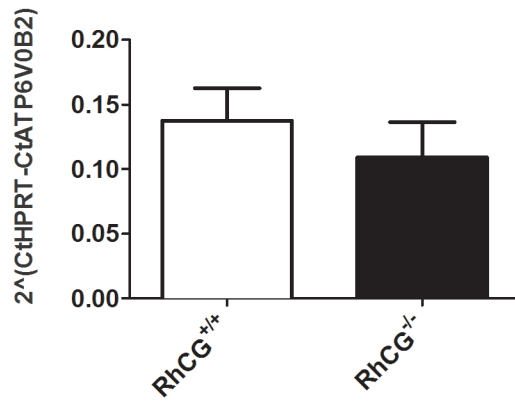
	Basal status		2 days HCl		4 days HCl	
	<i>C57BL6</i> (n=7)	<i>Atp6v1b1</i> <sup>-/-</sup> (n=7)	<i>C57BL6</i> (n=7)	<i>Atp6v1b1</i> <sup>-/-</sup> (n=7)	<i>C57BL6</i> (n=7)	<i>Atp6v1b1</i> <sup>-/-</sup> (n=5)
Weight (g)	22.7 ± 0.28	28.1 ± 0.5 <sup>#</sup>	20.4 ± 0.2*	24.6 ± 0.3 <sup>*#</sup>	20.5 ± 0.3*	23.0 ± 0.3 <sup>*#</sup>
Weight lose in % of body weight under basal status	0.1 ± 0.6	0.4 ± 0.9	10.3 ± 0.5*	13.0 ± 1.6 <sup>*#</sup>	11.9 ± 3.4*	18.8 ± 2.7 <sup>*#</sup>
Food intake (g/24hrs/body weight)	0.36 ± 0.01	0.36 ± 0.02	0.28 ± 0.01*	0.22 ± 0.03*	0.34 ± 0.04	0.26 ± 0.01 <sup>*#</sup>
Water intake (ml/24hrs/body weight)	0.17 ± 0.05	0.05 ± 0.01 <sup>#</sup>	0.20 ± 0.05	0.20 ± 0.04*	0.18 ± 0.03	0.23 ± 0.06*
Urine values						
volume (ml/24 h)	2.6 ± 0.4	3.0 ± 0.1	2.1 ± 0.2	4.7 ± 0.8 <sup>#</sup>	3.1 ± 0.2	3.9 ± 1.2
creatinine excretion (μmol/24 h)	3.7 ± 0.6	3.4 ± 0.2	3.4 ± 0.4	1.8 ± 0.3 <sup>*#</sup>	1.9 ± 0.2*	1.9 ± 0.7*
Urinary pH	6.2 ± 0.0	6.5 ± 0.1 <sup>#</sup>	5.4 ± 0.1*	6.0 ± 0.0 <sup>*#</sup>	5.7 ± 0.1*	6.0 ± 0.1 <sup>*#</sup>
UNH <sub>4</sub> /UCr (mEq/mmol)	3.4 ± 0.3	11.3 ± 1.2 <sup>#</sup>	51.0 ± 2.0*	56.7 ± 13.8*	88.9 ± 6.2*	76.6 ± 7.2 <sup>*#</sup>
UPi/UCr (mEq/mmol)	13.1 ± 1.1	11.6 ± 0.7	14.3 ± 2.4	18.8 ± 3.2	16.4 ± 2.6	22.7 ± 5.2

\* p<0.05 vs baseline period in same genotype, <sup>#</sup> p<0.05 vs *Atp6v1b1*<sup>+/+</sup> mice during the same period.

**Supplementary table 3. Primers and probes sequences of the gene expressions evaluated by RT-qPCR**

Gene	Accession number	Forward primer	Reverse primer	Probe
<b>Mouse</b> <i>Atp6v1b1</i>	NM_134157	5'- CCCAGTATGCTGAGATTGTCAACTTTAC CCTCC -3'	5'- CCTGAACAATGGCCTTGGTC-3'	5'- CCCAGTATGCTGAGATTGTCAACTTTAC CCTCC-3'
<b>Mouse</b> <i>Atp6v1b2</i>	NM_007509	5'- GCTGGTGATCCTGACGGACA TGAG-3'	5'- CGTGCCGGTCGAGTGGAAGGTAGAAAC GG-3'	5'- CTGCGAGAGGTTTCAGCTG-3'
<b>Mouse</b> <i>Atp6V0a4</i>	NM_080467	5'- GACGGTGTCTCATCTATGGTTTGA-3'	5'- TTTGCCCTGCATGGTCTTGTC-3'	5'- TCTCTCCATTATCAAGAGCTCATGCCA - 3'
<b>Mouse</b> <i>Hprt</i>	NM_013556	5'- TTATCAGACTGAAGAGCTACTGTAAGAT C-3'	5'- TTACCAGTGTCAATTATATCTTCAACAAT C-3'	5'- TGAGAGATCATCTCCACCAATAACTTTT ATGTCCC-3'
<b>Human</b> <i>RhCG</i>	NM_016321	5'- CTACCGACGCAACCTAGAGC-3'	5'-GCTGTCCCCATGGTAGGATA-3	5'-CTTTGCCATGATTGGCACCC-3'
<b>Human</b> <i>ATP6V1B1</i>	NM_001692	5'-GAGATGATTCAGACGGGCATTT-3'	5'-CTGGTGAAGAAGTCCAAGGC-3'	5'-AATGAGATTGCCGCTCAGAT-3'
<b>Human</b> <i>ATP6V1B2</i>	NM_001693	5''-TGAAGGGACTTCAGGTATAGATGC-3'	5'-GGAAGTCTTCGGCCAGTACA-3'	5'-TGGTCGGGTATTCAATGGAT-3'
<b>Human</b> <i>ATP6V0A1</i>	NM_001130020	5'-TTCCTGGAAGTACCGAATTA-3'	5'-AGGAGTGCCTCTTCCCATCT-3'	5'-GAGGCTGAATTGCATCATCA-3'
<b>Human</b> <i>HPRT</i>	NM_000185	5'-AAGGGTGTTACGTTTAT-3'	5'-TATTCCTGTGCGTGAT-3'	5'- ACCTCCTTGGGATCTATTCACCG -3'



**A****B****C**